

Extrusion Chemistry of Wheat Flour Proteins: I. Free Radical Formation

K. M. Schaich^{1,2} and C. A. Rebello^{1,3}

ABSTRACT

Cereal Chem. 76(5):748–755

Electron paramagnetic resonance (EPR) spectroscopy was used to study free radical production in hard red wheat flours extruded according to a two-level fractional factorial experimental design (11 and 14% protein content, 160 and 185°C, 16 and 20% moisture, 300 and 500 rpm screw speed, and mass flow rate of 225 and 400 g/min). All spectra showed dominant broad singlets ($g = 2.0053$ – 2.0059) from nitrogen-centered radicals originating from heat-induced peptide scission and reactions of lipid radicals with side-chain amino groups. At 77 K, sulfur-oxyl or peroxy radicals ($g = 2.008$ – 2.018), thiyl radicals ($g = 2.025$), and disulfide radi-

cal species ($g = 2.032$ – 2.035 and 2.05 – 2.06), resulting from intra- and intermolecular electron migration and shear-induced scission of disulfides, sometimes were present. The strongest EPR signals occurred under conditions of maximum free radical production and minimum opportunity for radical recombination: high protein flour (14%), high die temperature (180°C), and low moisture (16%). EPR signals correlated with sulfhydryl and disulfide (SH-SS) levels and physical properties of extrudates, indicating that free radicals are integrally involved in molecular changes that occur during extrusion.

Extrusion has become an important process for providing food products with a wide variety of textures. Texturization in extruded food materials has been attributed to a combination of fragmentation and aggregation (Armstrong et al 1979), noncovalent associations (Jeunink and Cheftel 1979, Areas 1986 1992), and covalent cross-linking (Burgess and Stanley 1976, Hurrell and Carpenter 1977, Hager 1984, Stanley 1989) of proteins and starches. However, despite extensive research on the physical properties of extruded materials and molecular changes that occur during extrusion, relatively little is understood yet at the molecular level, and extrusion remains largely an art rather than a science.

Electron paramagnetic resonance (EPR), also known as electron spin resonance (ESR), can provide direct evidence of the types and concentrations of radicals present in materials. EPR has been used for decades to study free radicals and paramagnetic metals in chemical systems and biological tissues (Swartz et al 1972), but its application to foods remains largely unexplored. Preliminary studies using EPR to detect free radicals in extruded cornmeal products revealed singlet signals with g values of 2.0053 – 2.0060 , which are typical of nitrogen-centered radicals (Fig. 1; Schaich 1990). The signals showed line shapes and intensities that varied with extrusion conditions and were nearly identical to EPR signals of radicals in dry proteins (Schaich and Karel 1976; Schaich 1980a–c). However, the specific origin, nature, and relevance of these free radicals could not be identified from the materials available at that time.

The existence of a free radical population, particularly one with sufficient stability or concentration to be detected by EPR (10^{11} spins or $10^{-9}M$; Borg 1976), indicates that bond breakage has occurred and predicts the likelihood of molecular degradation or cross-linking. The question is, what can EPR signals actually reveal about product chemistry during extrusion? Are EPR signals simply an artifact reflecting known bond breakage in doughs, or can they provide evidence that will help us understand the relevance and implications of free radicals in molecular changes induced by extrusion?

To gain more information about free radical production during extrusion and the possible relationship of free radicals to product quality, a systematic study of free radical production in wheat flour extrudates was undertaken. We report the quantification of free radical production in wheat flour as a function of extrusion conditions (temperature, moisture content of feed, and specific mechanical en-

ergy [SME]) and a comparison of heat, shear, and lipid oxidation as sources of free radicals. Correlation of extrudate free radicals with protein changes (protein content, sulfhydryl-disulfide [SH-SS] content, and structural changes) in extrudates are reported in an accompanying paper (Rebello and Schaich 1999).

MATERIALS AND METHODS

Two commercial wheat flours were obtained from Bay State Milling Co. (Minneapolis, MN): Bouncer (14% protein, 0.52% ash, 14% moisture) and Boss (11.4% protein, 0.41% ash, 14% moisture). Both flours were mixtures of hard red wheat cultivars. Bouncer was composed of spring wheats, and Boss was composed of mixed winter wheats containing up to 50% Bouncer. These flours were chosen because they are commonly used in bread flours and we wanted to compare their behavior in extrusion. Protein levels were selected to bracket (low and high) the useful functional range shown by Faubion and Hosney (1982a,b) for such flours.

Crude gluten (>60%) was purchased from Sigma Chemical Co. (St. Louis, MO). Hylon 7 (high amylose cornstarch) and amioca (amylopectin cornstarch) were obtained from National Starch and Chemical Co. (Bridgewater, NJ). High-purity methyl linoleate (ML) was obtained from Nu-Chek Prep (Elysian, MN).

Extrusion Conditions

Extrusion was conducted with a twin-screw extruder (ZSK30, Werner and Pfleiderer, Ramsey, NJ) equipped with two corotating, self-wiping screws with an outer diameter (D) of 30.7 mm, a length (L) of 878 mm, and an L:D ratio of 28.6 mm. The screw configuration (Fig. 2) consisted of forward-conveying elements (L:D = 21.9 mm), two mixing elements (L:D = 2.7 mm), six kneading blocks (L:D = 3.6 mm), and two reverse screw elements (L:D = 1.1 mm). The extruder had five barrel zones. The temperature in each zone was independently controlled by a combination of resistive electric heaters and cooling water jackets. The extruder die had two circular openings (3.6 mm diameter, 5 mm long). Product temperature and pressure at the die were measured with a flush probe (melt-pressure temperature probe model TPT 463E, Dynisco, Sharon, MA). Thermocouple probes were J-type (iron-constantin) junctions mounted flush in the ports at the die plate, which was located after the fifth zone of the extruder (Karwe and Godavarti 1997).

Wheat flour was fed through the feed port into the feed section by a precalibrated loss-in-weight feeder (series 7100, K-Tron Corp., Pittman, NJ). Water was injected into the feeding section immediately after the feed port with a calibrated triple-action piston pump (U.S. Electric Motors, Milford, CT), as described by Godavarti and Karwe (1997). After equilibration of the extruder, samples of extrudate from each extrusion run were collected, cooled, packed in glass jars, flushed with argon, sealed, and frozen until further use.

¹ Department of Food Science, Rutgers University, 65 Dudley Rd., New Brunswick, NJ 08901-8520.

² Corresponding author. Phone: 732/932-9611, ext. 233; Fax: 609/497-9313; E-mail: schaich@aesop.rutgers.edu

³ Current address: Campbell Soup Company, Campbell Place, Camden, NJ 08103.

The investigation was part of a larger cooperative project to study extrusion of puffed cereal products (Kokini et al 1992). Sample designations G37–G51 are internal codes for individual runs in one experiment of the project. Wheat flours were extruded according to a five-variable, two-level fractional factorial experimental design (Box et al 1978). The five independent extrusion variables were die temperature, screw speed, percent moisture, percent protein content of wheat flour, and mass flow rate. Variable levels were determined by preliminary experiments to be the practical limits of extrusion conditions for puffed products from wheat flour with the equipment used. Flours could not be extruded at moisture levels <16%, and textures began to collapse at moisture levels >20%. At temperatures lower than 160°C, starch was incompletely gelatinized, and at temperatures higher than 185°C, extrudates burned.

The dependent response variable SME provided a measure of mechanical stresses experienced by materials during passage through the extruder. SME is the amount of mechanical energy (work) dissipated as heat in the extruded material per unit mass of material (Godavarti and Karwe 1997). SME is measured as the work input from the drive motor into the material extruded and is strongly dependent on process conditions, such as screw speed, barrel temperature, moisture content, and feed composition, that affect viscosity and flow field in screw channels.

SME was derived from the mass flow rate and screw speed:

$$\text{SME (kJ/kg)} = (\text{mechanical energy input/kg material}) / (\text{torque} \times \text{angular screw speed/mass flow rate})$$

where angular screw speed is $2\pi(\text{rpm})$. The ZSK-30 extruder was equipped with a torque indicator that reported percent torque relative to direct current drawn by the drive motor; 100% torque corresponded to a maximum allowable torque of 172 Nm. The torque indicator and all other sensors (e.g., flow rate, temperature, pressure) were connected electronically to a data acquisition system (Keithley Metrabyte, Taunton, MA) that directly calculated SME from measured torque readings, as described by Godavarti and Karwe (1997):

$$\text{SME (kJ/kg)} = [(\text{total torque} - \text{friction torque}) \times N \times 9.1] / (172 \times 500 \times m_f)$$

where N is screw speed (revolutions/min); 9.1 is rated power (kW) of the drive motor at a rated screw speed of 500 rev/min; 172 is maximum allowable torque, and m_f is total mass flow rate (kg/sec) through the extruder barrel.

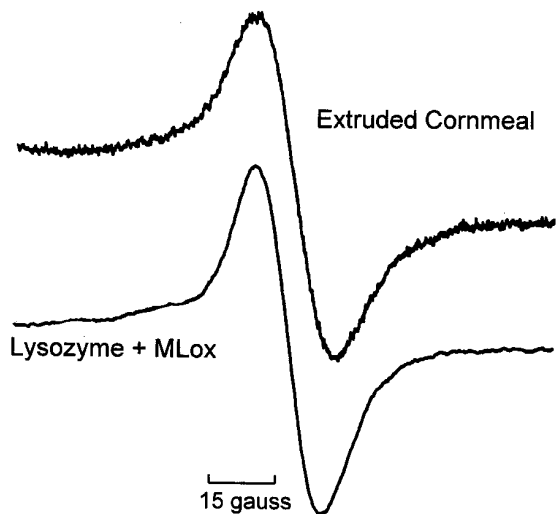


Fig. 1. Electron paramagnetic resonance (EPR) signals from extruded cornmeal show a central singlet with $g = 2.0053\text{--}2.0060$ (typical of nitrogen-centered radicals delocalized on proteins). EPR signals from extruded cornmeal are identical to those from dry lysozyme exposed to oxidizing methyl linoleate, showing a common property of electron delocalization on the peptide backbone. Although not marked, lysozyme has comparable g values.

EPR Measurement of Free Radicals

Samples of extrudates were removed from the freezer, crushed gently to homogeneity in a mortar and pestle, transferred to tared 10-mm i.d. quartz EPR tubes, and packed by tapping the bottom of the tube. Samples were packed to a height of ≈ 7 cm, which was sufficient to completely fill the EPR cavity. Samples were weighed, and packed heights were measured to calculate the packed density (g/cm³) of each sample. Tubes were capped and frozen in liquid nitrogen.

EPR analyses of extrudates were conducted at liquid-nitrogen (77 K) temperature to eliminate nonresonant effects of water on free radical signals (Bolton et al 1972) and to detect thiyl and disulfide radicals that often cannot be detected at room temperature. Large diameter tubes were used to increase sensitivity and because the low density and electrostatic surfaces of extrudates prevented packing in conventional 4-mm tubes. At least four replicate samples were analyzed for each extrusion run.

EPR analyses were conducted on a Varian E-12 EPR spectrometer interfaced to and controlled by a MassComp 5500 minicomputer and equipped with an X-band (9.5 GHz) microwave bridge, 100 kHz modulation, and variable rate signal averaging. Microwave frequencies were measured by a Hewlett Packard 505C frequency counter; magnetic field scans were routinely calibrated with a solution of Fremy's salt (peroxylamine disulfonate). The measurement temperature necessary for detection of sulfur radicals (77 K) was maintained with a Hewlett Packard variable temperature controller and liquid nitrogen/gas transfer assembly connected to a dewar in

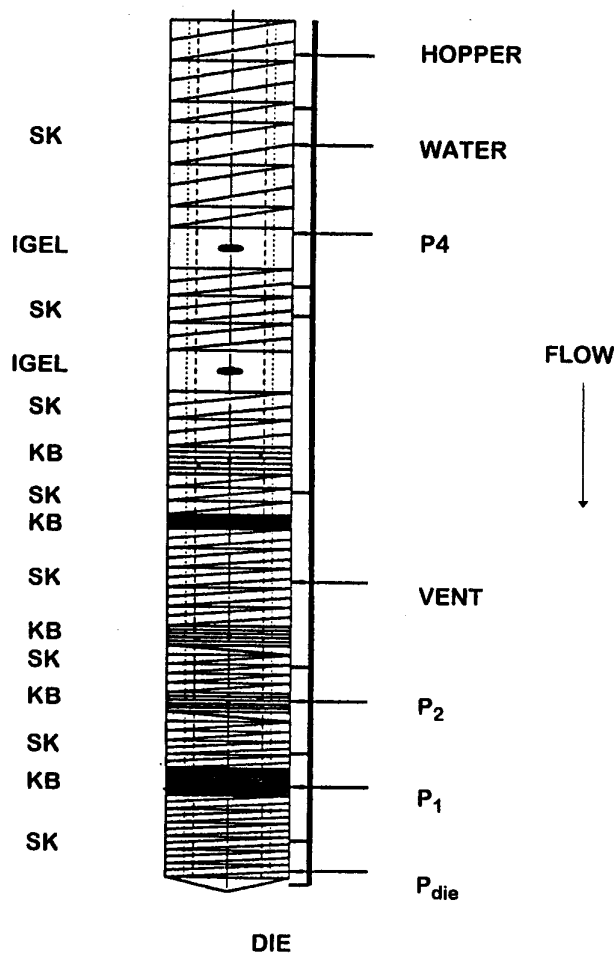


Fig. 2. Screw configuration for extrusion of wheat flour in a Werner and Pfleiderer ZSK30 twin-screw extruder. SK are high-pitch conveying elements; IGEL are porcupine mixing elements; KB are kneading blocks; P_x are pressure and temperature measurement points.

a wide-stack Endor cavity. Spectra were generated by repeatedly collecting and signal averaging 30-sec scans over at least 10 min and were presented as first derivatives of absorption at resonance (Bolton 1972, Bolton et al 1972). A diphenylpicrylhydrazyl (DPPH) crystal mounted in the cavity provided an internal field marker for normalizing successive scans. Data were collected, stored, and analyzed by computer.

Free radicals contributing to the spectra were identified by their g values, the proportionality constant characteristic and specific for each type of radical. The g value relates the microwave frequency and the resonant magnetic field of a radical and is calculated according to $h\nu = g\beta H$, where h is Planck's constant (6.625×10^{-27} erg sec), ν is the microwave frequency of the cavity (Hz, measured by frequency counter), and β is the Bohr magneton (9.274×10^{-21} erg gauss $^{-1}$) (Bolton 1972). H , the magnetic field at resonance, was determined from the field offset of the spectrum cross-over of the sample signal (determined from the second derivative) relative to the cross-over of either a solution or crystal of the stable radical DPPH ($g = 2.0037 \pm 0.0002$).

Signal intensities were calculated by double integration of experimental first-derivative spectra between the points at which each spectrum returned to baseline after corrections for baseline drift, and they were normalized to constant instrumental settings and packing density (g material/cm packed height) (Randolph 1972).

Spectrometer settings used for quantitative analyses were 5G modulation amplitude, 10mW power, 10^4 gain, 0.03 time constant, 200G field scan. Settings were below the level of modulation broadening and power saturation that distort signals. Gain was adjusted when necessary to keep the signal on scale, and final results were normalized to 10,000 gain.

To determine the effects of heat in the absence of shear stress, flour (14% moisture) was spread evenly over the bottom of a petri dish and heated in a laboratory oven at 180°C. Samples were withdrawn at various intervals, packed in 4-mm i.d. EPR tubes, and EPR spectra were recorded at room and liquid-nitrogen temperatures as described above. As with extrudates, signal intensities were

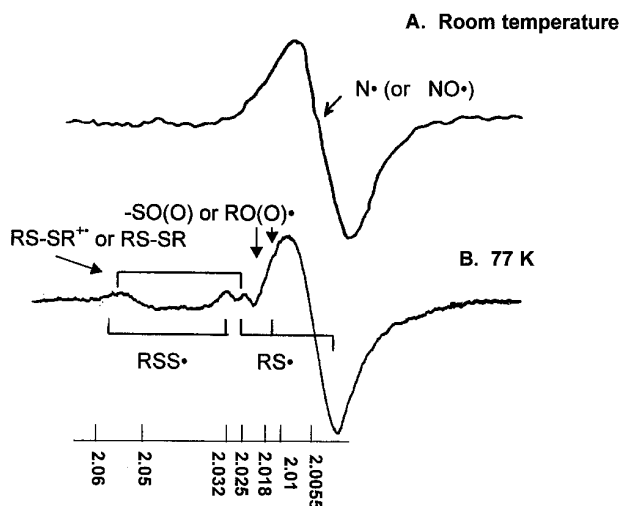


Fig. 3. A, Typical room-temperature electron paramagnetic resonance (EPR) signal from extruded wheat flours. All samples showed a central broad singlet, with $g = 2.0053$ – 2.0059 , from nitrogen-centered radicals delocalized on proteins. B, Liquid-nitrogen (77 K) EPR signal showing various spectral components observed in extruded wheat flours. All samples showed a central singlet from nitrogen radicals ($g_{av} = 2.0055$). Some samples had shoulders at $g \approx 2.01$ and 2.02 attributable to peroxy and sulfoxyl radicals, respectively, as well as additional peaks ($g \approx 2.025$, 2.032 , and 2.05 – 2.06) to the left of the central singlet that arise from various sulfur radicals. The intensity of the central peak and appearance of sulfur and peroxy peaks varied with extrusion conditions.

normalized to constant packing density and expressed as arbitrary free radical units per gram of material.

Radical production by oxidizing lipids was tested by mixing methyl linoleate (ML) with flour, gluten, or starch at 1:5 and 1:10 (by weight) ratios and incubating at room temperature in a desiccator over CaSO_4 . Samples were withdrawn periodically, packed in 4-mm i.d. EPR tubes, and EPR spectra were recorded at room and liquid-nitrogen temperatures. Use of standard 4-mm tubes was necessary in the heating and oxidation experiments and with unextruded controls because the dense flour in larger diameter tubes shifted the microwave frequency in the sample cavity excessively.

Statistics

Correlations between EPR signal intensity and extrusion conditions, chemical changes, and physical properties were calculated by statistics programs in Excel software (Microsoft Corp.).

RESULTS AND DISCUSSION

Free Radical Production by Extrusion

No unextruded flour samples showed distinguishable, quantifiable EPR signals at room temperature; at 77 K, metal signals with free radical components too weak for quantitation were detected (data not shown). Therefore, free radical signals detected in extrudates resulted entirely from the extrusion process.

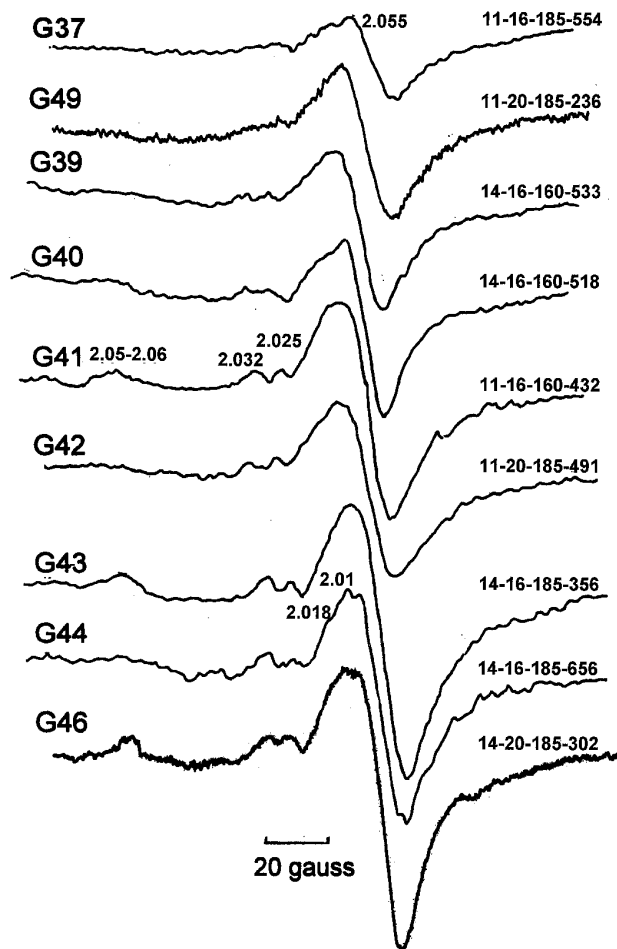


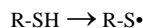
Fig. 4. Representative electron paramagnetic resonance (EPR) signals at 77 K from extruded Boss (11.4% protein) and Bouncer (14% protein) hard red wheat flours. Notations to the right of each spectrum indicate extrusion conditions: protein content-moisture-temperature-SME (%-%-°C-kJ/kg). G37–G51 refer to extrusion run number (listed in Table I).

EPR spectra from wheat flour extrudates revealed multiple radical species and were very similar to EPR signals from dry proteins reacted with oxidizing lipids (Schaich and Karel 1976, Schaich 1980c), exposed to ionizing radiation and photolysis (Schaich 1980a,b), or damaged by mechanical force (Redman et al 1966, Wasik and Bushuk 1973, Chandra and Symons 1987).

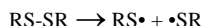
At room temperature, dry extrudates produced only relatively broad single-line spectra with g values of 2.0053–2.0059 (Fig. 3A), indicating nitrogen-centered radicals (Bolton et al 1972).

When extrudates were analyzed at 77 K, peaks arising from several types of radicals appeared (Figs. 3B and 4). Under all conditions, the dominant feature of the spectra was the strong central line from nitrogen radicals ($g = 2.0049$ – 2.0059 , $>N\bullet$ and $>NO\bullet$ radicals). Shoulders with $g \approx 2.008$ – 2.01 to the left of the central peak typically arise from peroxy radicals (Bersohn and Thomas 1964), e.g., on proteins or perhaps carbohydrates in the extrudates. Shoulders with $g \approx 2.018$ – 2.022 are consistent with sulfur oxyl radicals ($RSO\bullet$ or $RSO\bullet$) (Gilbert et al 1975, Sevilla et al 1987).

Three sets of peaks indicative of sulfur radicals were observed: $g \approx 2.025$, $g \approx 2.032$, and $g = 2.05$ – 2.06 . When present, the peaks were variable in both proportion and intensity, reflecting multiple sulfur radical species in different conformations and environments. Peaks at $g \approx 2.025$ are common to nearly all species of sulfur radicals observed in dry sulfhydryl proteins, amino acids, and small thiol compounds exposed to ionizing radiation (Henriksen and Pihl 1961, Henriksen et al 1976) or reacted with oxidizing lipids (Schaich and Karel 1976, Schaich 1980c). This is the dominant central line of spectra generally attributed to thiyl radicals formed by hydrogen abstraction from cysteine:



or scission of disulfide bonds (Kurita and Gordy 1961):



although there has been some disagreement about the assignment (Symons 1974). Combinations of peaks with $g \approx 2.025$, 2.032 , and 2.05 – 2.06 arise from disulfide radicals. Signals with $g = 2.024$ and 2.05 – 2.07 have been attributed to $RSS\bullet$ radicals in irradiated proteins (Hadley and Gordy 1974). $RSS\bullet$ may form during extrusion by scission of the C-S bond, which is almost as weak as the S-S bond (259 vs. 226 kJ/mol, respectively; Masterton and Hurley

1989). Signals with central lines at $g = 2.024$ – 2.038 have been ascribed to $RS\bullet$ -SR, $(RS-SR)^+$ and $(RS-SR)^-$ species formed by recombination of two $RS\bullet$ radicals (Symons 1974, Nelson et al 1977) or oxidation or reduction of disulfide compounds (Box et al 1970) and vary with the orientation as well as the nature of the radicals. It also has been proposed that $g = 2.025$ and 2.032 lines are actually splittings of a single line from a protonated disulfide species, $RS\bullet$ -SHR (Chandra and Symons 1987). Considering the line shapes and variability in extrudate spectra, as well as the forces present during extrusion, it is reasonable to propose that more than one of these species are present in extrudates simultaneously and that there are some interconversions between species with time.

The total signal intensity and proportion of sulfur radicals varied with extrusion conditions (Fig. 4; Table I). The highest total free radical concentration and sulfur radical production occurred at high extrusion temperature (185°C) and low moisture content (16%) in high protein (14%) flour (Table I). The lowest stable free radical concentrations were found in samples extruded at a lower temperature (160°C) and higher moisture content (20%).

The two flours showed distinct differences in free radical correlations with extrusion parameters. With the higher protein flour Bouncer, moisture and temperature had strong effects on free radical production ($\rho = -0.67$ and 0.55 , respectively), but shear (SME) had little influence ($\rho = 0.11$). In contrast, SME had a moderate effect on radical production ($\rho = 0.46$) in the lower protein flour Boss, whereas moisture and temperature had weak or no correlations ($\rho = -0.26$ and 0.01 , respectively).

These results with wheat flour support previous observations (Maurice and Stanley 1978) that protein concentration and extrusion temperature were the key factors in texturization of extruded soy flour and that texturization effects of extrusion parameters were marked in high protein flours while textures in low protein flours were relatively independent of extrusion conditions. However, further research is needed to clarify the differential effects of protein type and composition versus protein concentration on extrusion.

Free Radical Production by Heat

EPR signal characteristics and correlations of signal intensity with extrusion temperature suggested that heat may be the dominant radical-producing force during extrusion. To test this possibility,

TABLE I.
Experimental Extrusion Design and Relationship Between Extrusion Conditions and Electron Paramagnetic Resonance (EPR) Signal Intensity

Sample	Protein (%)	Moisture (%)	Die Temp. ($^\circ\text{C}$)	Screw Speed (rpm)	Mass Flow (g/min)	SME ^a (kJ/kg)	EPR Signal Intensity (arbitrary units)	S ^b	SO ^b	(S-S) ^{+b,c}
Bouncer										
G45 ^d	14	20	185	500	400	356	1,410 \pm 85			
G46	14	20	185	300	225	302	5,554 \pm 1,592	++	+	++
G51	14	20	160	500	225	512	1,793 \pm 122			
G50	14	20	160	300	400	356	1,318 \pm 113	+	(+)	
G44	14	16	185	500	225	656	6,823 \pm 174	++	++	(+)
G43	14	16	185	300	400	356	8,973 \pm 1,956	++		++
G40	14	16	160	500	400	518	4,771 \pm 290	+	+	+
G39	14	16	160	300	225	533	3,460 \pm 279	+	(+)	(+)
ρ (EPR) ^e	0.25	-0.67	0.55			0.11				
Boss										
G42 ^d	11.4	20	185	500	400	491	4,901 \pm 503	++	++	
G49	11.4	20	185	300	400	236	1,540 \pm 108			
G47	11.4	20	160	500	400	410	1,704 \pm 99			
G38	11.4	16	185	500	225	1,016	4,492 \pm 214	+	+	(+)
G37	11.4	16	185	300	400	554	1,580 \pm 305	(+)	+	
G48	11.4	16	160	500	225	416	3,108 \pm 151	+	+	
G41	11.4	16	160	300	225	432	4,652 \pm 514	++	+	++
ρ (EPR)	0.25	-0.26	0.01			0.46				

^a Specific mechanical energy.

^b Relative intensity. Visual estimation of presence of designated sulfur radical species in EPR signal. ++ = strong; + = moderate; and (+) = present, but weak.

^c Multiple species of disulfide radicals present.

^d G37–G51 are extrusion run numbers. Data are presented in a sequence that facilitates comparisons, not in the order in which experiments were run.

^e Correlation of individual extrusion variables with EPR signal intensity.

free radical production was monitored in Bouncer flours, gluten, and starches heated at 180°C. Materials were heated dry to isolate the effects of heat and to eliminate influences of water on radical recombination and stability.

EPR signals from heated flour were nitrogen-centered singlets ($g = 2.0060$; Fig. 5A) and increased with heating time (Fig. 5B). Spectral envelopes in heated flour were narrower than in flour extruded or reacted with oxidizing lipids (discussed below), indicating a more homogeneous population of radicals with high localization of free electrons within the solid flour matrix.

There was no evidence of sulfur radicals in either room- or low-temperature EPR spectra of flour heated dry. Typical sulfur spectra still were not detected when up to 20% moisture was added during heating. It is possible that sulfur radicals formed but were unstable during heating. However, the free radical data were consistent with chemical analyses showing no changes in thiol or disulfide contents in heated gluten until moisture content exceeded 20% (Hansen et al 1975, Weegels et al 1994).

Flour is a mixture of starch and protein components, so gluten and starch were heated separately to evaluate their potential contributions to flour spectra. All gluten and starch samples heated at 180°C produced singlet EPR signals with $g \approx 2.0057$ (Fig. 5A) from nitrogen-centered radicals, as was observed in spectra from extrudates. EPR signal levels for isolated gluten were much stronger than those for flour (Fig. 5B). In contrast, EPR signals in starch were very weak (Fig. 5B). The trace levels of nitrogen-centered radicals detected in starch probably originated from protein or other nitrogenous contamination of starch. Likely sources of contamination include surface and integral proteins associated with the starch granule, which are exceedingly difficult to remove completely from starch preparations. Wheat starch, for example, typically contains 0.4–0.6% protein (Sulaiman and Morrison 1990).

Carbon-centered radicals from starch bond scission would have shown up as separate lines or peaks at $g = 2.002$ – 2.003 , but no such peaks were distinguishable in any starch spectra. Whether free radicals from starch are not observed because starch molecules do not undergo heat-induced scission or, more likely, because starch radicals decay or react too rapidly for detection is being investigated.

These results show that the EPR signals in wheat flour extrudates arise from component proteins and further suggest that heat may be the major source of free radicals from the peptide backbone and the major contributor to central nitrogen radical signal

intensity. Nevertheless, heat alone cannot account for the broadness of lines or for the sulfur radical components of EPR signals from extruded wheat flours.

Free Radical Production by Oxidizing Lipids

In addition to the physical forces of heat and shear, a potential chemical source of free radical production during flour extrusion is lipid oxidation. Although the lipid content of wheat flour is very low, the component fatty acids are predominately unsaturated (Chung 1991) and susceptible to oxidation when exposed to heat, oxygen, and metals during extrusion. Lipid alkoxy and peroxy radicals can transfer radicals to amino acid side chains with nitrogen and sulfur functional groups (Schaich and Karel 1976, Schaich 1980c).

Typical EPR signals produced in flour and flour components by reaction with oxidizing ML (ML = 5:1 in flour) are shown in Fig. 6. Spectral envelopes were much broader than those from heated flours, typical of overlapping spectra from multiple nitrogen radical sources in different environments (Borg 1976). There was no clear evidence of sulfur radicals, even in low-temperature spectra.

Previous studies have shown that oxidizing lipids only transfer free radicals to surface sulfhydryl groups on proteins and cannot react with disulfides unless proteins are denatured to expose these bonds (Schaich and Karel 1976). Inaccessibility of thiol and disulfide groups to lipid radical species in unmodified wheat flour may explain the absence of sulfur radical signals in these model systems. However, proteins become denatured during extrusion, exposing reactive groups, so some sulfur radical production by reaction with oxidizing lipids could occur during extrusion.

When oxidizing ML was incubated with commercial cornstarch, amioca, and Hylon 7 starches for comparison, only very weak signals were detected, with g values of 2.0056. As in the heating experiment, these signals probably arose from protein or other nitrogenous contaminants in the starch preparation.

Origins of EPR Free Radical Signals from Extrudates

In considering the implications of these results, it is important to note that EPR does not provide a virtual record of free radical production. Rather, EPR spectra reflect only free radicals that remain stable over the time of detection. Radicals present in very low concentrations, with very short life times (msec), or with very broad lines in their spectra may not be detected in static measurements, such as those used in our study. Because static EPR mea-

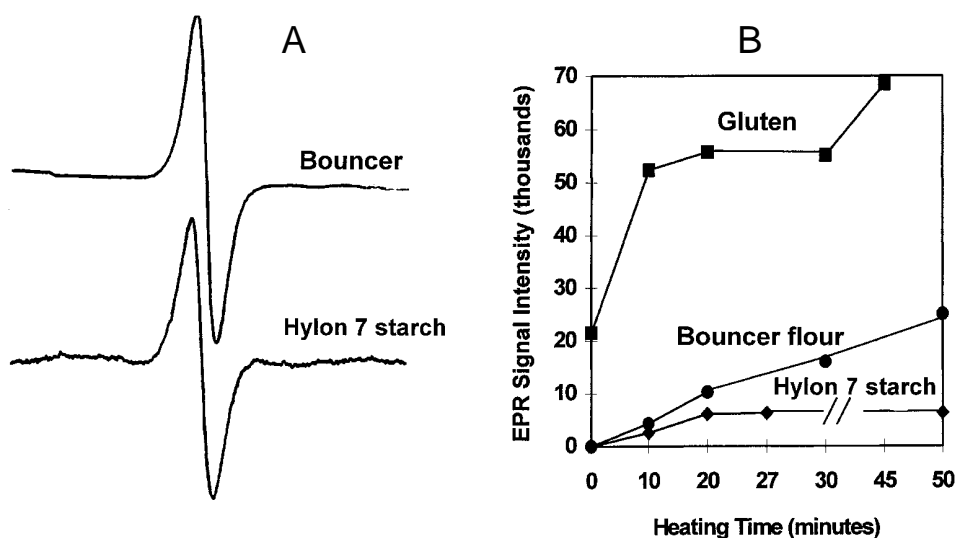
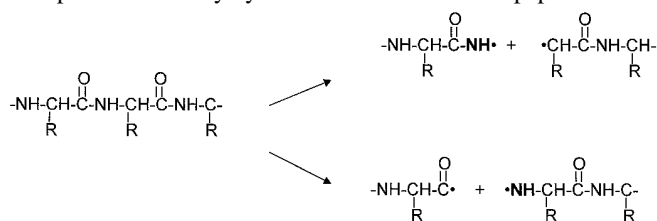


Fig. 5. A, Electron paramagnetic resonance (EPR) signal of dry Bouncer flour and Hylon 7 starch (amylose) heated at 180°C for 30 min. Starch signal ($g = 2.0063$) is enlarged to reveal line shape. **B,** Free radical production as a function of heating time for Hylon 7 starch, Bouncer flour, and isolated gluten. Gluten produced an EPR signal that included background metals before heating; the free radical component of the signal increased with heating time.

measurements cannot directly reflect the radical history of a sample, free radicals that are produced and recombine, react, or otherwise convert to nonradical species are not detected and, consequently, must be deduced from product analyses. Also, EPR spectra of solids are not as definitive as solution spectra because they are composite spectra of radicals fixed in multiple orientations. Nevertheless, the technique is very powerful and can provide significant information about free radical production and stabilization.

In the present study, multiple radical species were apparent in EPR spectra from wheat flour extrudates, including nitrogen-centered radicals, a variety of sulfur radical species, and perhaps peroxy radicals. Results of model system studies provide plausible explanations for the origins of various spectral components.

The *g* values of the EPR signals, comparisons with EPR spectra already published, and spectra generated in model systems indicate that signals in wheat flour extrudates arise from proteins in the flour and that multiple forces contribute to production of radicals during extrusion. The quantitation of EPR signals in heated materials and strong correlation between EPR signals and die temperature suggest that the nitrogen-centered radicals that dominated the spectra were produced mainly by heat-induced scission of peptide chains:



Direct demonstration of peptide free radicals induced by extrusion and heat in our study provides a mechanistic explanation for changes observed in other studies of extruded and heated proteins. Cumming et al (1973) reported that heat during extrusion led to breakdown of protein and loss of water solubility. Hansen et al (1975) found that heating proteins at temperatures higher than 150°C induced peptide and disulfide bond scission, release of low molecular weight peptides, and destruction of lysine and arginine. Destruction of cystine-cysteine occurred only when moisture content was >20%. Hager (1984) proposed that temperature was the major factor leading to protein insolubilization during thermal processing of protein solutions. The critical relationship between heat and moisture in the generation and fate of free radicals in proteins is clear in all these studies, and the results are consistent with a mechanism involving heat-induced formation of radicals by peptide scission, followed by radical recombination to generate intermolecular cross-links.

In addition to the central singlet from nitrogen radicals, specific downfield peaks (left of the central line) typical of sulfur radicals appeared in EPR spectra of some extruded samples. Because EPR signals of sulfur radicals were not detected in flour heated or reacted with oxidizing lipids, these processes may not be major sources of sulfur radicals, which leaves shear stress or radical transfer reactions as the most reasonable alternatives for production of sulfur radicals during extrusion. Disulfide bonds have the lowest bond energy in protein chains: S-S (226), C-C (347), C-N (293), C-S (259), and S-H (339), all in kJ/mol, at 25°C (Masterton and Hurley 1989). As a result, they provide a weak link that can be severed by shear force.

There is a precedent for proposing scission of disulfide bonds by shear force. Axford et al (1962), Dronzek and Bushuk (1968), and MacRitchie (1975) suggested that high-speed mixing of doughs would break disulfide bonds and form thiyl radicals. EPR detection of sulfur radicals after mechanical disruption of α-keratin (a disulfide protein) verified that shear stress was the probable mechanism (Chandra and Symons 1987). Also, sonication to increase solubilization of wheat flour proteins selectively depolymerized disulfide bonds in high molecular weight glutenins, with no apparent effect on other protein classes (Singh et al 1990).

The results of the present study support a relationship between shear force, disulfide scission, and free radical production. Sulfur radicals were strongest in samples extruded at the highest shear force and absent or weak at low shear. Statistically, there were very strong correlations between total EPR signal strength and SH and SS contents of extrudates (Rebello and Schaich 1999). Decrease in disulfide and increase in sulfhydryl contents were associated with high free radical content. Conversely, increased disulfides (from sulfur radical recombinations) occurred in extrudates with lower free radical contents (Rebello and Schaich 1999).

However, the relationship was not straightforward. Bouncer extrudates had the strongest sulfur resonance in EPR signals, but SME did not significantly affect EPR signal strength or thiol-disulfide changes. In Boss extrudates, although there were weak or no sulfur radical peaks in the EPR signals, the correlation between shear stress and free radical content was reasonably strong (*p* = 0.46), and the effect of SME on thiol-disulfide contents was significant (Rebello and Schaich 1999).

Several factors may explain the anomaly. One factor may be the inherent reactivity of sulfur compounds and radicals: sulfur radicals are formed but rapidly decay, oxidize, or recombine into non-radical products during extrusion. Also, although Boss is lower in protein content, its disulfide content is slightly higher, so the gliadins or glutenins in Boss may be more susceptible to shear stress. Another factor confounding the correlation between shear stress and sulfur radical generation is that shear force in extrusion is a dependent variable. Shear force results from material friction and is controlled by the interactions of several primary variables, including moisture content, die temperature, and mass flow rate. Lack of direct correlation also may be attributed to the contradictory effects

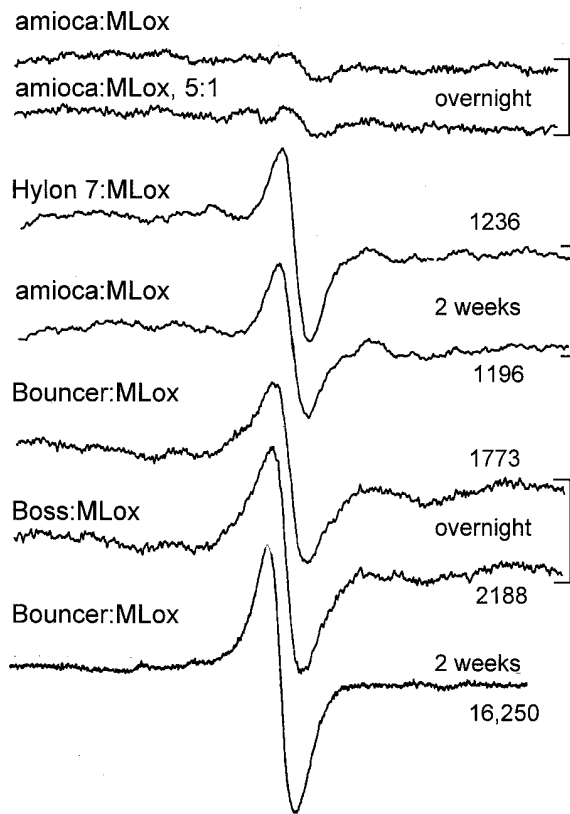


Fig. 6. Room-temperature electron paramagnetic resonance (EPR) signals induced in flour and starch by incubation with oxidizing methyl linoleate for varying time periods. Numbers to the right indicate signal intensity in arbitrary units per gram of material, normalized to constant sample packing. For starch, overnight flour, and two-week flour samples, *g* = 2.0052, 2.0055, and 2.0059, respectively. Signals are not scaled proportionally in presentation.

of shear: shear breaks disulfide bonds, but low and high shear rates also increase the possibility of contact between free radicals on adjacent protein molecules. At low shear, the bond is broken, but the two radicals do not move far enough apart to prevent reformation. At high shear, the free radical ends of multiple broken bonds are moved rapidly past each other, increasing the opportunity for intermolecular encounter and recombination. At moderate shear, the radicals ends may move so far apart that they cannot reconnect, but they are limited enough in movement that they do not encounter other molecules.

Finally, shear may not be the only source of sulfur radicals during extrusion. The high electronegativity of sulfur makes it a strong electron sink. Thus, sulfur-centered radicals in extrudates may be generated by transfer of free electrons from peptide or lipid oxy radicals to thiols and disulfides, generating $RS\cdot$ and $RS\cdot-SR$ or $(RS-SR)^{\cdot-}$, respectively (Symons 1974). Alternatively, oxidizing radicals, such as $HO\cdot$, $RO\cdot$, or $ROO\cdot$, may pull electrons from disulfide bonds, yielding $(RS-SR)^{\cdot+}$ (Bonifacic and Asmus 1976). In the present study, sulfur radical signals were strongest under extrusion conditions of high nitrogen radical production (high heat) and low radical recombination (low moisture), but only low to moderate shear. These extrusion conditions also favor lipid oxidation, which is another source of radicals that react with cysteine, cystine, and sulfur proteins (Schaich and Karel 1976).

When taken together, the data suggest that shear stress contributes to scission of disulfide bonds during extrusion and that the resulting radicals may be involved in cross-linking reactions during extrusion. However, shear stress may not be the major source of stable and EPR-detectable sulfur radicals in extruded materials.

The broad line shapes of the EPR spectra make it clear that the radicals present are not highly localized in an ordered environment and that heat-induced peptide radicals are not the only radicals present in extrudates. Oxidation of some peptide radicals to protein peroxy radicals is certainly a possibility that must be investigated. However, it also is likely that alkoxy and peroxy radical intermediates of oxidizing lipids generate nitrogen free radicals via hydrogen abstraction from amino acid side chains. Wheat flour reacted with up to 10% (weight basis) ML developed free radical signals with line shapes that very closely matched EPR signals from extrudates. This type of signal broadening occurs because lipid-induced side-chain radicals on lysine, histidine, arginine, and tryptophan of proteins all are nitrogen-centered but differ slightly in g values and hyperfine structure (Schaich and Karel 1976). The individual spectra of the various component radicals overlap to broaden and add shoulders to the main nitrogen radical signal of extrudates. The extrudates did not contain added lipid, but wheat flour has $\approx 1-2\%$ lipid content, of which the major fatty acid components, oleic and linoleic acids (Chung 1991), are unsaturated and susceptible to oxidation. Thus, it is reasonable to expect that lipid-mediated radical attack on proteins may occur during extrusion and contribute to free radical production on proteins.

Summary and Conclusions

EPR free radical signals were detected and quantitated in wheat flour extruded under various conditions, providing the first direct evidence that protein free radicals are produced during extrusion. Nitrogen-centered and sulfur radicals are produced in wheat flour during extrusion, and extrusion die temperature and moisture, as well as flour protein content, interact to influence the production and subsequent reactions of these free radicals. The net radical population producing EPR signals in wheat flour extrudates resulted from a complex balance between factors producing free radicals (i.e., heat, shear stress, protein concentration, lipid oxidation) and factors favoring radical reaction or recombination (i.e., moisture, shear stress, lower temperature). Shifts in dominant free radical species and recombination mechanisms under different extrusion conditions seem likely and may explain variations in texture characteristics.

EPR signals of free radicals in extrudates are not simply inter-

esting artifacts of the extrusion process. Correlations of free radical production with chemical changes in wheat flour proteins and physical properties of extrudates (Rebello and Schaich 1999) indicate that free radicals are integrally involved in protein changes during extrusion. The fact that the addition of free radical scavengers during extrusion of wheat flour prevents protein cross-linking and markedly alters extrudate structures (Koh et al 1996) further substantiates the critical function of free radicals in extrusion.

Stanley (1989) stated that "A knowledge of the way in which proteins are rebonded is necessary for understanding the mechanism of texturization" in extrusion. The recognition of a key role for free radicals in extrusion chemistry opens many new avenues for research that should contribute significantly to understanding general processes of texturization and structure formation at the molecular level in foods.

ACKNOWLEDGMENTS

We express our thanks and gratitude to Mukund Karwe, who supervised the extrusion of samples. This publication No. D-10544-11-98 of the New Jersey Agricultural Experiment Station was supported by New Jersey State Funds and the Center for Advanced Food Technology (CAFT). CAFT is a New Jersey Commission on Science and Technology Center.

LITERATURE CITED

- Areas, J. A. G. 1986. Hydrophobic and electrostatic interactions in extrusion of protein isolates. *J. Food Sci.* 51:1311-1313,1322.
- Areas, J. A. G. 1992. Extrusion of food proteins. *CRC Crit. Rev. Food Sci. Nutr.* 32:365-392.
- Armstrong, D. L., Maurice, T. J., and Stanley, D. W. 1979. Functional properties of microwave-heated soybean proteins. Pages 147-172 in: *Functionality and Protein Structure*. A. Pour-El, ed. Am. Chem. Soc.: Washington, DC.
- Axford, D. W. E., Campbell, J. D., and Elton, G. A. H. 1962. Changes in apparent disulfide content of doughs on mechanical development. *Chem. Ind. (Lond.)* 407-408.
- Bersohn, M., and Thomas, J. R. 1964. Identification of peroxy radicals by EPR. *J. Am. Chem. Soc.* 86:959.
- Bolton, J. R. 1972. Electron spin resonance theory. Pages 11-62 in: *Biological Applications of Electron Spin Resonance*. H. M. Swartz, J. R. Bolton, and D. C. Borg, eds. Wiley-Interscience: New York.
- Bolton, J. R., Borg, D. C., and Swartz, H. M. 1972. Experimental aspects of biological electron spin resonance. Pages 63-118 in: *Biological Applications of Electron Spin Resonance*. H. M. Swartz, J. R. Bolton, and D. C. Borg, eds. Wiley-Interscience: New York.
- Bonifacic, M., and Asmus, K.-D. 1976. Free radical oxidation of organic disulfides. *J. Phys. Chem.* 80:2426-2430.
- Borg, D. C. 1976. Applications of electron spin resonance in biology. Pages 69-147 in: *Free Radicals in Biology*, Vol. 1. W. A. Pryor, ed. Academic Press: New York.
- Box, G. E. P., Hunter, W. G., and Hunter, J. S. 1978. Fractional factorial design at two levels. Pages 374-418 in: *Statistics for Experimenters*. John Wiley & Sons: New York.
- Box, H. C., Freund, H. G., Lilga, K. T., and Budzinski, E. E. 1970. Magnetic resonance studies of the oxidation and reduction of organic molecules by ionizing radiation. *J. Phys. Chem.* 74:40-52.
- Burgess, L., and Stanley, D. W. 1976. A possible mechanism for thermal texturization of soybean protein. *Can. Inst. Food Sci. Technol. J.* 9:228-231.
- Chandra, H., and Symons, M. C. R. 1987. Sulphur radicals formed by cutting alpha-keratin. *Nature* 328:833-834.
- Chung, O. K. 1991. Cereal lipids. Pages 497-554 in: *Handbook of Cereal Science and Technology*. K. J. Lorenz and K. Kulk, eds. Marcel Dekker: New York.
- Cumming, D. B., Stanley, D. W., and deMan, J. M. 1973. Fate of water soluble soy protein during thermoplastic extrusion. *J. Food Sci.* 38:320-323.
- Dronzek, B., and Bushuk, W. 1968. A note on the formation of free radicals in dough during mixing. *Cereal Chem.* 45:286-288.
- Faubion, J. M., and Hoseney, R. C. 1982a. High-temperature short-time extrusion cooking of wheat starch and flour: I. Effect of moisture and flour type on extrudate properties. *Cereal Chem.* 59:529-533.
- Faubion, J. M., and Hoseney, R. C. 1982b. High-temperature short-time ex-

- trusion cooking of wheat starch and flour: II. Effect of protein and lipid on extrudate properties. *Cereal Chem.* 59:533-537.
- Gilbert, B. C., Laue, A. H., Norman, R. O. C., and Sealy, R. C. 1975. Electron spin resonance studies: Part XLVI: Oxidation of thiols and disulfides in aqueous solution: Formation of $RS\bullet$, $RSO\bullet$, $RSO_2\bullet$, $RSSR^-$, and carbon radicals. *J. Chem. Soc. Perkin Trans. II.* 892-900.
- Godavarti, S., and Karwe, M. V. 1997. Determination of specific mechanical energy distribution on a twin-screw extruder. *J. Agric. Eng. Res.* 67: 277-287.
- Hadley, J. H., and Gordy, W. 1974. Nuclear coupling of ^{33}S and the nature of free radicals in irradiated crystals of cystine dihydrochloride. *Proc. Natl. Acad. Sci. USA* 71:3106-3110.
- Hager, D. F. 1984. Effects of extrusion upon soy concentrate solubility. *J. Agric. Food Chem.* 32:293-296.
- Hansen, L. P., Johnston, P. H., and Ferrel, R. E. 1975. Heat-moisture effects on wheat flour: I. Physical-chemical changes of flour proteins resulting from thermal processing. *Cereal Chem.* 52:459-472.
- Henriksen, T., Melo, T. B., and Saxebo, G. 1976. Free radical formation in proteins and protection from radiation damage. Pages 213-256 in: *Free Radicals in Biology*. Vol. 2. W. A. Pryor, ed. Academic Press: New York.
- Henriksen, T., and Pihl, A. 1961. The migration of radiation damage in reduced glutathione. *Int. J. Radiat. Biol.* 3:351-359.
- Hurrell, R. F., and Carpenter, K. J. 1977. Nutritional significance of cross-link formation during heat processing. Pages 225-238 in: *Protein Cross-linking: Nutritional and Medical Consequences*. Part 2. M. Friedman, ed. Plenum Press: New York.
- Jeunink, J., and Chefel, J. C. 1979. Chemical and physicochemical changes in field bean and soybean proteins texturized by extrusion. *J. Food Sci.* 44:1322-1325.
- Karwe, M. V., and Godavarti, S. 1997. Accurate measurement of extrudate temperature and heat loss on a twin-screw extruder. *J. Food Sci.* 62: 367-372.
- Koh, B. K., Karwe, M. V., and Schaich, K. M. 1996. Effects of cysteine on free radical production and protein modification in extruded wheat flour. *Cereal Chem.* 73:115-122.
- Kokini, J. L., Solberg, M., and Henriksen, F. 1992. A new model for food research: CAFT—Rutgers University. *Trends Food Sci. Technol.* 3:128-132.
- Kurita, Y., and Gordy, W. 1961. Electron spin resonance in a gamma-irradiated single crystal of L-cysteine dihydrochloride. *J. Chem. Phys.* 34: 282-288.
- MacRitchie, F. 1975. Mechanical degradation of gluten proteins during high speed mixing of doughs. *J. Polymer Sci.* 49:85-90.
- Masterton, W. L., and Hurley, C. N. 1989. Covalent bond properties. Page 267 in: *Chemistry Principles and Reactions*. Saunders College Publishing: Philadelphia.
- Maurice, T. J., and Stanley, D. W. 1978. Texture-structure relationships in texturized soy protein. IV. Influence of process variables on extrusion texturization. *Can. Inst. Food Sci. Technol. J.* 11:1-6.
- Nelson, D. J., Petersen, R. L., and Symons, M. C. R. 1977. Unstable intermediates: Part 178: The structure of intermediates formed in the radiolysis of thiols. *J. Chem. Soc. Perkin Trans. II:*2005-2015.
- Randolph, M. L. 1972. Quantitative considerations in electron spin resonance studies in biological materials. Pages 119-154 in: *Biological Applications of Electron Spin Resonance*. H. M. Swartz, J. R. Bolton, and D. C., Borg, eds. Wiley-Interscience: New York.
- Rebello, C. A., and Schaich, K. M. 1999. Extrusion chemistry of wheat flour proteins: II. Sulfhydryl-disulfide content and structural changes. *Cereal Chem.* 86:756-763.
- Redman, D. G., Axford, D. W. E., Elton, G. A. H., and Briuati, J. A. 1966. Mechanically produced radicals in flour. *Chem. Ind. (Lond.)* 1298-1302.
- Schaich, K. M. 1980a. Free radical initiation in proteins and amino acids by ionizing and ultraviolet radiations and lipid oxidation: Part I: Ionizing radiation. *CRC Crit. Rev. Food Sci. Nutr.* 13:89-129.
- Schaich, K. M. 1980b. Free radical initiation in proteins and amino acids by ionizing and ultraviolet radiations and lipid oxidation: Part II: Ultraviolet radiation and photolysis. *CRC Crit. Rev. Food Sci. Nutr.* 13:131-159.
- Schaich, K. M. 1980c. Free radical initiation in proteins and amino acids by ionizing and ultraviolet radiations and lipid oxidation: Part III: Free radical transfer from oxidizing lipids. *CRC Crit. Rev. Food Sci. Nutr.* 13:189-244.
- Schaich, K. M. 1990. Chemical changes during extrusion. Physical forces in food systems (Dec. 1990). Pages 70-75 in: *Research Accomplishments of the Center for Advanced Food Technology*. Rutgers University: New Brunswick, NJ.
- Schaich, K. M., and Karel, M. 1976. Free radical reactions of peroxidizing lipids with amino acids and proteins: An ESR study. *Lipids* 11:392-400.
- Sevilla, M. D., Becker, D., Swarts, S., and Herrington, J. 1987. Sulfinyl radical formation from the reaction of cysteine and glutathione thyl radicals with molecular oxygen. *Biochem. Biophys. Res. Commun.* 144:1037-1042.
- Singh, N. K., Donovan, G. R., Batey, I. L., and MacRitchie, F. 1990. Use of sonication and size-exclusion high-performance liquid chromatography in the study of wheat flour proteins: I. Dissolution of total proteins in the absence of reducing agents. *Cereal Chem.* 67:150-161.
- Stanley, D. W. 1989. Protein reactions during extrusion processing. Pages 321-341 in: *Extrusion Cooking*. C. Mercier, P. Linko, and J. M. Harper, eds. Am. Assoc. of Cereal Chem.: St. Paul, MN.
- Sulaiman, B. D., and Morrison, W. R. 1990. Proteins associated with the surface of wheat starch granules purified by centrifuging through cesium chloride. *J. Cereal Sci.* 12:53-61.
- Swartz, H. M., Bolton, J. R., and Borg, D. C., eds. 1972. *Biological Applications of Electron Spin Resonance*. Wiley-Interscience: New York.
- Symons, M. C. R. 1974. On the electron spin resonance detection of $RS\bullet$ radicals in irradiated solids: Radicals of type $RSSR^-$, $RS-SR_2$, and $R_2SSR_2^+$. *J. Chem. Soc. Perkin Trans. II:*1618-1620.
- Wasik, R. J., and Bushuk, W. 1973. Free radicals in flour, starch, and gluten produced by ball-milling, electric discharge, and gamma-irradiation. *Cereal Chem.* 50:654-660.
- Weegels, P. L., de Groot, A. M. G., Verhoek, J. A., and Hamer, R. J. 1994. Effects on gluten of heating at different moisture contents: II. Changes in physico-chemical properties and secondary structure. *J. Cereal Sci.* 19:39-47.

[Received August 14, 1998. Accepted May 26, 1999.]